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(54) Title: **USE OF AMINO-OXY FUNCTIONAL GROUPS IN THE PREPARATION OF VACCINES**

(57) Abstract: The invention relates to a process for preparing a conjugate comprising combining an amino-oxy homofunctional or heterofunctional reagent with an entity chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules containing at least one carbonyl group, to form a polysaccharide, oligosaccharide, carbohydrate, or carbohydrate-containing molecule functionalized via at least one oxime linkage. The functionalized compound is then reacted either directly or indirectly with a protein moiety to form a protein-carbohydrate conjugate that may be used as a vaccine.

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Use of Amino-Oxy Functional Groups in the Preparation of Vaccines

This application claims benefit of priority of U.S. Provisional Application Nos. 60/539,573 filed January 29, 2004, and 60/589,019, filed July 20, 2004.

Background of the invention

5 The present invention relates to a process of covalently linking proteins and polysaccharides to form conjugate vaccines comprising a reaction between carbonyl-containing groups and amino-oxy functional groups.

 In the process of vaccination, medical science uses the body's innate ability to protect itself against invading agents by immunizing the body with antigens that
10 will not cause the disease but will stimulate the formation of antibodies that will protect against the disease. For example, dead organisms are injected to protect against bacterial diseases such as typhoid fever and whooping cough, toxoids are injected to protect against tetanus and diphtheria, and attenuated organisms are injected to protect against viral diseases such as poliomyelitis and measles.

15 It is not always possible, however, to stimulate antibody formation merely by injecting the foreign agent. The vaccine preparation must be immunogenic, that is, it must be able to induce an immune response. Certain agents such as tetanus toxoid can innately trigger the immune response, and may be administered in vaccines without modification. Other important agents are not
20 immunogenic, however, and must be converted into immunogenic molecules or constructs before they can induce the immune response.

 The immune response is a complex series of reactions that can generally be described as follows: (1) the antigen enters the body and encounters antigen-presenting cells that process the antigen and retain fragments of the antigen on
25 their surfaces; (2) the antigen fragments retained on the antigen-presenting cells are recognized by T cells that provide help to B cells; and (3) the B cells are

stimulated to proliferate and divide into antibody forming cells that secrete antibody against the antigen.

Most antigens only elicit antibodies with assistance from the T cells and, hence, are known as T-dependent (TD). Examples of such T-dependent antigens
5 are tetanus and diphtheria toxoids.

Some antigens, such as polysaccharides, cannot be properly processed by antigen presenting cells and are not recognized by T cells. These antigens do not require T cell assistance to elicit antibody formation but can activate B cells directly and, hence, are known as T-independent antigens (TI). Such T-
10 independent antigens include *H. influenzae* type b polyribosyl-ribitol-phosphate (PRP) and pneumococcal capsular polysaccharides.

There are other differences between T-independent and T-dependent antigens.

A) T-dependent antigens, but not T-independent antigens, can prime an
15 immune response so that a memory response results on secondary challenge with the same antigen.

B) The affinity of the antibody for antigen increases with time after immunization with T-dependent, but not T-independent antigens.

C) T-dependent antigens stimulate an immature or neonatal immune
20 system more effectively than T-independent antigens.

D) T-dependent antigens usually stimulate IgM, IgG1, IgG2a, and IgE antibodies, while T-independent antigens stimulate IgM, IgG1, IgG2b, and IgG3 antibodies.

T-dependent antigens can stimulate primary and secondary responses,
25 which are long-lived in both adult and in neonatal immune systems, but must frequently be administered with adjuvants (substances that enhance the immune

response). Very small proteins, such as peptides, are rarely immunogenic, even when administered with adjuvants.

T-independent antigens, such as polysaccharides, are able to stimulate immune responses in the absence of adjuvants, but cannot stimulate high level or
5 prolonged antibody responses. They are also unable to stimulate an immature or B cell defective immune system (Mond, J. J., *Immunological Reviews*, 64:99 (1982); Mosier, D. E. et al., *J. Immunol.*, 119:1874 (1977)).

For T-independent antigens, it is desirable to provide protective immunity against such antigens to children, especially against capsular polysaccharides
10 found on organisms such as *H. influenzae*, *S. pneumoniae*, and *Neisseria meningitidis*.

One approach to enhance the immune response to T-independent antigens involves conjugating polysaccharides such as *H. influenzae* PRP (Cruse, J. M., Lewis, R. E. Jr., eds., *Conjugate Vaccines in Contributions to Microbiology and*
15 *Immunology*, Vol. 10, (1989)), or oligosaccharide antigens (Anderson, P. W. et al., *J. Immunol.*, 142:2464, (1989)) to a T-dependent antigen such as tetanus or diphtheria toxoid. Recruitment of T cell help in this way has been shown to provide enhanced immunity to many infants that have been immunized.

Protein-polysaccharide conjugate vaccines stimulate an anti-
20 polysaccharide antibody response in infants who are otherwise unable to respond to the polysaccharide alone.

Conjugation of a protein and a polysaccharide may provide other advantageous results. For example, Applicant has found that a protein/polysaccharide conjugate may enhance the antibody response not only to
25 the polysaccharide component, but also to the protein component. This effect is described, for example, in U.S. Patent No. 5,955,079. This effect also is described in A. Lees, et al., *Vaccine*, 12(13):1160 (1994).

Techniques have been developed to facilitate coupling of proteins and polysaccharides. See, for example, Dick, W. E. et al., "Glyconjugates of Bacterial Carbohydrate Antigens: A Survey and Consideration of Design and Preparation Factors," *Conjugate Vaccines* (Eds. Cruse, et al.), p. 48 (1989). Many techniques
5 for activation of carbohydrates, however, are not suitable for use in aqueous media because the activating or functional reagents are not stable in water. For example, N,N'-carbonyldiimidazole, as described in Marburg et al., U.S. Patent No. 4,695,624, must be used in organic media.

Homofunctional and heterofunctional vinylsulfone reagents have been used
10 to activate polysaccharides. The activated polysaccharides are reacted with a protein, peptide, or hapten, under appropriate reaction conditions, to produce the conjugate. This is described in more detail in U.S. Patent No. 6,309,646. Another method for producing conjugate vaccines comprises mixing a uronium salt reagent with a soluble first moiety, such as a polysaccharide or carbohydrate, and
15 combining therewith a second moiety, such as a protein, peptide, or carbohydrate, to form the conjugate vaccine. This method is described in U.S. Patent No. 6,299,881.

Most carbohydrates must be activated before conjugation, and cyanogen bromide (CNBr) is frequently the activating agent of choice. See, e.g., Chu et al.,
20 *Inf. & Imm.*, 40:245 (1983). The first licensed conjugate vaccine was prepared with CNBr to activate HIB PRP, which was then derivatized with adipic dihydrazide and coupled to tetanus toxoid using a water-soluble carbodiimide.

The use of 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate, also called "CDAP," has been described for use in aqueous media to activate
25 polysaccharides. These activated polysaccharides may be directly or indirectly coupled to proteins. The use of CDAP is described in, for example, U.S. Patent No. 5,849,301 and in Lees, et al., "Activation of Soluble Polysaccharides with 1-

Cyano-4-Dimethylamino Pyridinium Tetrafluoroborate For Use in Protein-Polysaccharide Conjugate Vaccines and Immunological Reagents," *Vaccine*, 14(3):190 (1996).

To briefly summarize the CNBr-activation method, CNBr is reacted with the
5 carbohydrate at a high pH, typically a pH of 10 to 12. At this high pH, cyanate esters are formed with the hydroxyl groups of the carbohydrate. These, in turn, are reacted with a bifunctional reagent, commonly a diamine or a dihydrazide. These derivatized carbohydrates may then be conjugated via the bifunctional group. In certain limited cases, the cyanate esters may also be directly reacted to
10 protein.

The high pH is necessary to ionize the hydroxyl group because the reaction requires the nucleophilic attack of the hydroxyl ion on the cyanate ion (CN⁻). As a result, CNBr produces many side reactions, some of which add neo-antigens to the polysaccharides. Wilcheck, M. et al., *Affinity Chromatography. Meth.*
15 *Enzymol.*, 104:3-55 (1984). More importantly, many carbohydrates or moieties such as Hib, PRP, and capsular polysaccharides from and pneumococcal type 6 and *Neisseria meningitis* A can be hydrolyzed or damaged by the high pH necessary to perform the cyanogen bromide activation.

Another problem with the CNBr activation method is that the cyanate ester
20 formed is unstable at high pH and rapidly hydrolyzes, reducing the yield of derivatized carbohydrate and, hence, the overall yield of carbohydrate conjugated to protein. Many other nonproductive side reactions, such as those producing carbamates and linear imidocarbonates, are promoted by the high pH. This effect is described in Kohn et al., *Anal. Biochem*, 115:375 (1981). Moreover, CNBr itself
25 is highly unstable and spontaneously hydrolyzes at high pH, further reducing the overall yield.

Protein-polysaccharide conjugate vaccines may also be formed via reductive amination. In this method, aldehydes on the polysaccharide are reacted with amines on the protein to form a reversible Schiff base. The Schiff base is subsequently reduced to form a stable linkage between the amine and the aldehyde. This process is beset by a number of problems. The formation of the Schiff base is slow and inefficient, and the overall reaction is further impeded by the large size of the two components (i.e., the polysaccharide and protein), which need to be in close proximity with each other in order to react. In order to overcome this problem, the polysaccharide is often broken down into oligosaccharides prior to coupling.

The use of dimethylsulfoxide (DMSO) promotes the formation of the Schiff base, but this organic solvent can harm the protein. Sometimes a multistep protocol is used, in which a spacer group (e.g., hexane diamine or adipic dihydrazide) is added to the polysaccharide via reductive amination, and this spacer is subsequently ligated to the protein. Using a high concentration of the spacer helps to force the reaction and increase the yield. Elevated temperatures and prolonged reaction times are also used to promote the reaction. However, these can also be detrimental to the protein and the polysaccharide. Furthermore, as amines must be deprotonated to react with aldehydes, the Schiff base formation usually requires the use of alkaline solutions, i.e., solutions at a pH \geq 8. Prolonged reactions at elevated temperature and pH can be detrimental to both the protein and the polysaccharide. Furthermore, the reductive step, which usually involves the use of cyanoborohydride or pyridine-boranes, can be inefficient and deleterious to the protein. Also, these reagents can be hazardous to work with in large quantities. A further limitation of the reductive amination method is the highly random nature of the linkage sites between the protein and the polysaccharide.

Accordingly, there remains a need in the art for an efficient and effective process for preparing conjugate vaccines.

Summary of the Invention

One embodiment includes a process for preparing a conjugate vaccine,
5 comprising:

(a) reacting a first moiety containing at least one carbonyl-containing group with at least one amino-oxy reagent to form at least one pendent functional group on the first moiety, wherein the first moiety is chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules;

10 (b) reacting the first moiety containing at least one pendent functional group with a second moiety to form a composition comprising a conjugate, wherein the second moiety is chosen from proteins, peptides, and haptens; and

(c) combining the conjugate with a pharmaceutically acceptable delivery vehicle to form a conjugate vaccine.

15 Another embodiment includes a process for preparing a conjugate vaccine, comprising:

(a) reacting a first moiety containing at least one pendent amino-oxy group with a second moiety to form a composition comprising a conjugate,

(b) wherein the first moiety is chosen from polysaccharides,
20 oligosaccharides, carbohydrates, and carbohydrate-containing molecules, and the second moiety is chosen from proteins, peptides, and haptens; and

(c) combining the conjugate with a pharmaceutically acceptable delivery vehicle to form a conjugate vaccine.

Another embodiment includes a process for preparing a conjugate vaccine,
25 comprising:

(a) reacting a first moiety chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules, with

(b) a second moiety reacted with at least one amino-oxy reagent, wherein the second moiety is chosen from proteins, peptides, and haptens, to form a composition comprising a conjugate; and

(c) combining the conjugate with a pharmaceutically acceptable delivery
5 vehicle to form a conjugate vaccine.

Yet another embodiment includes a process for preparing a conjugate vaccine, comprising:

(a) reacting a first moiety with a second moiety containing at least one pendent amino-oxy group to form a composition comprising a conjugate,

10 wherein the first moiety is chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules, and the second moiety is chosen from proteins, peptides, and haptens; and

(b) combining the conjugate with a pharmaceutically acceptable delivery vehicle to form a conjugate vaccine.

15 A further embodiment includes a process for preparing a conjugate vaccine, comprising:

(a) providing a first moiety chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules;

(b) providing a second moiety chosen from N-terminal 1,2-aminoalcohols
20 which can be oxidized to contain at least one aldehyde group;

(c) functionalizing said second moiety with at least one amino-oxy reagent;

(d) reacting said first moiety with the functionalized second moiety to form a composition comprising a conjugate; and

(e) combining the conjugate with a pharmaceutically acceptable delivery
25 vehicle to form a conjugate vaccine.

A further embodiment includes a process for preparing a conjugate vaccine, comprising:

(a) reacting a first moiety containing at least one pendent amino-oxy group, wherein the first moiety is chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules;

(b) reacting the first moiety with a second moiety to form a composition
5 comprising a conjugate, wherein the second moiety is chosen from glycoproteins containing at least one carbonyl group; and

(c) combining the conjugate with a pharmaceutically acceptable delivery vehicle to form a composition comprising an conjugate vaccine.

Still another embodiment includes a process for preparing a conjugate
10 vaccine, comprising:

(a) reacting a first moiety chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules with a second moiety chosen from proteins, peptides, and haptens to form a composition comprising a conjugate.

15 (b) wherein the first moiety contains at least one reducing end derivatized with an amino-oxy reagent, and

(c) combining the conjugate with a pharmaceutically acceptable delivery vehicle to form a conjugate vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 is an SDS-page chromatogram showing a high degree of protein-polysaccharide conjugation.

Figure 2 is an SDS-page chromatogram showing BSA-polysaccharide conjugation.

Figure 3 shows the results of a resorcinol assay for protein and
25 carbohydrate of fractions eluting from an S-400HR™ (Pharmacia) gel filtration column.

Figure 4 shows an SDS-PAGE chromatogram indicating the occurrence of protein-polysaccharide conjugation.

Figures 5A-5D indicate the presence of higher molecular weight conjugates of fractions eluting from an S-400HR™ (Pharmacia) gel filtration column.

5 Figure 6 is an SDS-PAGE chromatogram showing the presence of conjugate fractions.

Figure 7 is a chromatogram comparing a conjugate with its unconjugated components.

Figure 8 illustrates the results of an opsonic assay.

10 **Definitions**

Amino-oxy reagent refers to a reagent with the structure $\text{NH}_2\text{-O-R}$. R can be any group capable of bonding to the amino-oxy nitrogen. According to one aspect of the disclosure, R is a functional group, e.g., an amine, thiol, or other chemical group facilitating coupling to, e.g., a protein.

15 **Conjugate** means to chemically link or join together.

Functionalize means to add at least one group that facilitates further reaction. Typical functional groups include amino-oxy, thiol, maleimide, halogen, haloacyl, aldehyde, hydrazide, hydrazine, and carboxyl. Other functional groups would be well known to the person of ordinary skill in the art and can be found
20 discussed in Hermanson, *Bioconjugation Techniques*.

Hapten refers to a small molecule such as a chemical entity that by itself is not able to elicit an antibody response, but can elicit an antibody response once it is coupled to a carrier.

Homofunctional, when discussing an amino-oxy reagent, refers to a
25 reagent that has at least two amino-oxy functional groups. The homofunctional agent may be homobifunctional or homomultifunctional, i.e., having two, three, four or more amino-oxy functional groups.

Heterofunctional, when discussing an amino-oxy reagent, refers to a reagent that has at least one amino-oxy functional group and at least one other non-amino-oxy functional group. The heterofunctional agent may be heterobifunctional or heteromultifunctional, i.e., having two, three, four or more amino-oxy functional groups. It may also have more than one other non-amino-oxy functional group, such as two, three, or four or more, of either the same type or different types.

Moiety refers to one of the parts of a conjugate.

Pendent functional group refers to a functional group that is exists on or is exposed on a molecule.

Spacer refers to an additional molecule that is used to indirectly couple the first moiety to the second moiety.

Detailed Description of the Invention

A. Strategy for Conjugation

The present invention provides an alternative to prior art processes for preparing conjugate vaccines. Specifically, the invention provides for new methods of conjugating a first moiety to a second moiety, where the first moiety is chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules and the second moiety is chosen from proteins, peptides, and haptens, and the conjugation proceeds using at least one amino-oxy functional group

There are a number of ways of reacting the first and second moiety within the scope of the invention and each of these methods rely on using at least one amino-oxy group in the process.

At least one amino-oxy reagent with one amino-oxy group may be reacted with the first moiety to form a composition with at least one non-amino-oxy pendent functional group.

At least one amino-oxy reagent with more than one amino-oxy group may be reacted with the first moiety to form a composition with at least one amino-oxy pendent functional group. In this embodiment, there may optionally additionally be present at least one non-amino-oxy pendent functional group.

- 5 At least one amino-oxy reagent with one amino-oxy group may be reacted with the second moiety to form a composition with at least one non-amino-oxy pendent functional group.

- At least one amino-oxy reagent with more than one amino-oxy group may be reacted with the second moiety to form a composition with at least one amino-oxy pendent functional group. In this embodiment, there may optionally additionally be present at least one non-amino-oxy pendent functional group.

- Thus, in this invention, at least one of the first moiety and the second moiety will be reacted with an amino-oxy reagent, and will result in a composition with at least one pendent functional group (at least one of an amino-oxy or non-amino-oxy pendent functional group). It is possible to functionalize both the first moiety and the second moiety according to any combination of strategies 1 or 2 (first moiety) and 3 or 4 (second moiety), as set forth immediately above. In another embodiment, either the first moiety or the second moiety may be functionalized.

- 20 The first moiety and the second moiety may then be conjugated together. This conjugation may proceed directly, by linking the pendent functional group on the first moiety directly to the second moiety. Alternatively, this conjugation may proceed indirectly, by linking the pendent functional group on the first moiety to an additional agent called a spacer, which is then linked to the second moiety.
- 25 Certainly, a similar strategy may be followed with a pendent functional group on the second moiety, simply by reversing the positions of the first and second moiety.

B. The First Moiety: Polysaccharide, Oligosaccharide, Carbohydrate, and Carbohydrate-Containing Molecules

As used herein, "carbohydrate" means any soluble monosaccharide, disaccharide, oligosaccharide, or polysaccharide. Examples of suitable polysaccharides for use in the process of the invention include bacterial, fungal, and viral polysaccharides. Soluble polysaccharides (i.e., polysaccharides present in solution), such as water-soluble polysaccharides, are suitable for use in accordance with the present invention. Specific examples of suitable polysaccharides include *Salmonella typhi* Vi antigen; *Neisseria meningitidis* polysaccharide C; and Pneumococcal polysaccharides, such as Pneumococcal polysaccharide type 14

According to certain embodiments of the present invention, the carbohydrate is naturally occurring, a semisynthetic, or a totally synthetic large molecular weight molecule. According to one embodiment, at least one carbohydrate-containing moiety is selected from *E. coli* polysaccharides, *S. aureus* polysaccharides, dextran, carboxymethyl cellulose, agarose, Pneumococcal polysaccharides (Pn), Ficoll, *Cryptococcus neoformans*, *Haemophilus influenzae* PRP, *P. aeruginosa*, *S. pneumoniae*, Group A and B streptococcus, *N. meningitidis*, and combinations thereof.

According to one embodiment, the carbohydrate-containing moiety is a dextran. As used herein, "dextran" (dex) refers to a polysaccharide composed of a single sugar, which may be obtained from any number of sources (e.g., Pharmacia). Another preferred carbohydrate-containing moiety is Ficoll, which is an inert, semisynthetic, non-ionized, high molecular weight polymer. Additional non-limiting examples of moieties that may be used in accordance with the present invention include lipopolysaccharides ("LPS"), lipooligopolysaccharides ("LOS"), lipotechoic acid ("LTA"), deacylated LPS, deacylated LTA, delipidated

LPS, delipidated LTA, and related molecules. Generally, a carbohydrate-containing molecule that has been coupled using reductive amination requires the formation of an aldehyde moiety. In those instances, for example, these aldehydes may also be coupled using amino-oxy chemistry described herein.

5 Reductive amination has been used to couple LPS and LOS, both of which can be coupled using amino-oxy chemistry. Examples of coupling of LPS and LOS using reductive amination chemistry may be found in Mieszala et al., *Carbohydrate Research*, 338:167 (2003); Jennings et al., *Inf. & Immun.*, 43:407 (1984); and U.S. Patent No. 4,663,160.

10 C. The Second Moiety: Proteins, Peptides and Haptens

In accordance with the present invention, various different proteins can be coupled to various different polysaccharides. The following list includes examples of suitable proteins that may be used in accordance with the invention: viral proteins, bacterial proteins, fungal proteins, parasitic proteins, animal proteins.

15 Glycoproteins from any of the above sources may also be used to form a conjugate with the first moiety. Lipids, glycolipids, peptides, and haptens are also suitable for use as a second moiety in this invention. Haptenated proteins, i.e., proteins derivatized with haptens, are also suitable for use as a second moiety in this invention.

20 Specific proteins include tetanus toxoid (TT), pertussis toxoid (PT), bovine serum albumin (BSA), lipoproteins, diphtheria toxoid (DT), heat shock protein, T-cell superantigens, protein D, CRM197, and bacterial outer-membrane protein. All of these protein starting materials may be obtained commercially from biochemical or pharmaceutical supply companies (e.g., American Tissue Type Collection in
25 Rockville, MD or Berna Laboratories of Florida) or may be prepared by standard methodologies, such as those described in J. M. Cruse and R. E. Lewis (Eds.),

"Conjugate Vaccines in Contributions to Microbiology and Immunology", Vol. 10 (1989).

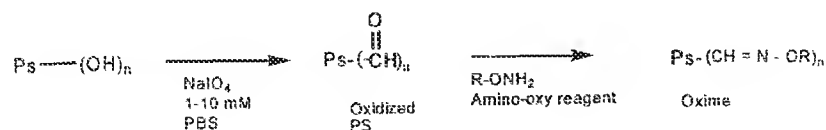
D. Methods for Functionalizing the First or Second Moiety with an Amino-Oxy Group

5 The amino-oxy (also referred to as oxy-amine, amino-oxy, aminooxy, and amino-oxy) functional group, $\text{NH}_2\text{-O-R}$, has a lower pKa than the amines found on proteins, and is nucleophilic at much lower pH. Amino-oxy groups react well with carbonyl-containing groups, e.g., aldehydes and ketones, to form highly stable oximes. The optimum pH for the reaction can range from 4 to 8, for example from
10 5 to 7. According to one aspect of the invention, the optimum pH is around 5. Since oximes are stable, the reductive step in the reductive amination process, discussed above, is optional. The high efficiency of the reaction may result in shorter reaction times. Furthermore, it is possible to exert some control over the reaction sites between the complementary reagents. By contrast, the reaction of
15 hydrazides and amines with groups such as, for example, ketones, is slower and far less efficient.

The protein and polysaccharide are functionalized with complementary oxime-forming groups, and reacted to form oxime-linked protein-polysaccharide conjugate vaccines. According to one aspect of the invention, the protein is
20 directly linked to the polysaccharide.

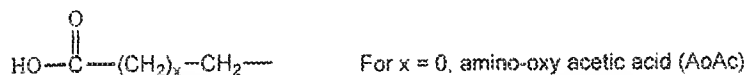
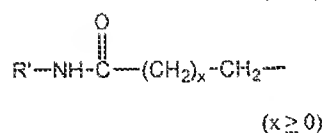
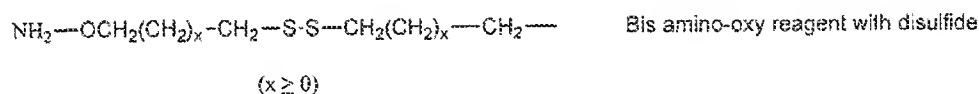
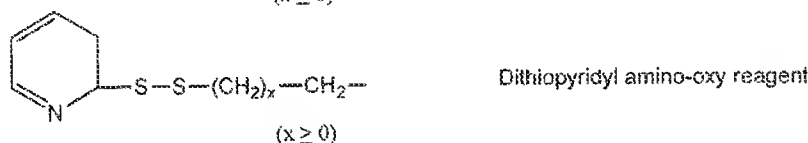
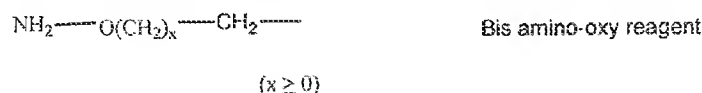
According to one embodiment, there is provided a process comprising combining an amino-oxy homofunctional or heterofunctional reagent with an entity chosen from polysaccharides, oligosaccharides, carbohydrates, and carbohydrate-containing molecules containing at least one carbonyl group, to form
25 a polysaccharide, oligosaccharide, carbohydrate, or carbohydrate-containing molecule functionalized via at least one oxime linkage. Functionalized means to add a group which facilitates further reaction, for example, thiol, carboxy, amino-

oxy, halogen, aldehydes, and the like. This embodiment may be illustrated by the following non-limiting illustration ("Ps" denotes a polysaccharide):



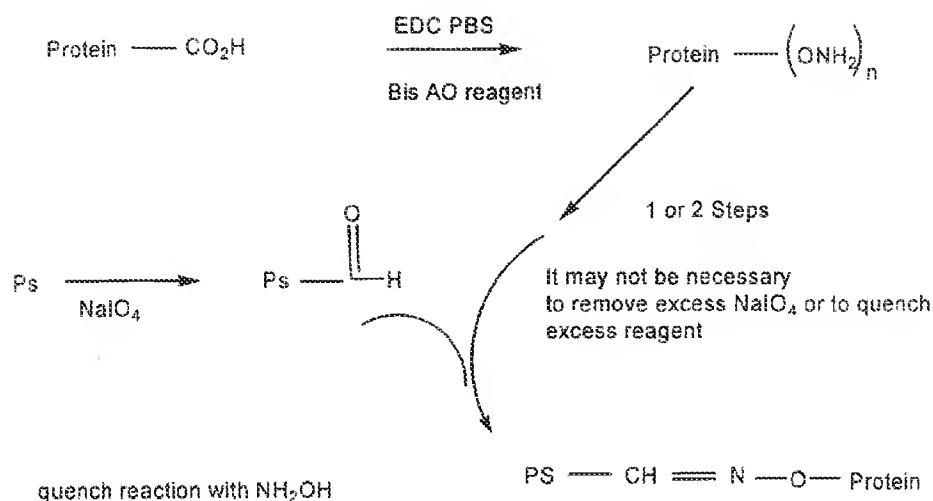
R is a functional group, e.g., an amino-oxy, amine, thiol, or other chemical group,

- 5 such as those listed below, for facilitating coupling to the protein:



The at least one pendent functional group is then reacted directly or indirectly with the protein moiety to yield a protein-polysaccharide conjugate.

- 10 According to another embodiment, the protein is functionalized with at least one pendent amino-oxy group, which is subsequently reacted with a carbonyl group on a polysaccharide, oligosaccharide, carbohydrate, or carbohydrate-containing moiety. The carbonyl group is formed with, for example, sodium periodate. For example, in the case of a polysaccharide, the functionalized
- 15 protein is reacted with the polysaccharide to form a protein-polysaccharide conjugate. The following scheme illustrates a non-limiting aspect of this process:



Methods for functionalizing a protein with an amino-oxy group are known to those of ordinary skill in the art. The protein can be functionalized with amino-oxy groups chemically, enzymatically or by genetic engineering. Described herein are methods for functionalizing the protein on either amines or carboxyl groups, and for controlling the number of amino-oxy groups on the protein.

In yet another embodiment, the polysaccharide is functionalized with pendent amino-oxy groups and subsequently reacted with a glycoprotein containing carbonyl groups. These may be present, for example, by oxidizing the carbohydrate on the glycoprotein. Aldehydes may be created by selective oxidation of N-terminal serine or threonine.

In accordance with the present invention, for example when the polysaccharide, oligosaccharide, carbohydrate, or carbohydrate-containing moiety is functionalized with an amino-oxy group, the protein advantageously contains at least one carbonyl group in the form of, e.g., a ketone or aldehyde moiety. Aldehydes may be created on proteins containing an N-terminal serine or threonine, and the resulting protein can be reacted with an amino-oxy reagent,

thus uniquely functionalizing the N-terminal. This monovalently-functionalized protein can then be reacted directly, for example, with a carbonyl-containing polysaccharide, if the amino-oxy reagent is homofunctional or indirectly, using spacers. N-terminal serine or threonine can occur naturally, or be engineered into
5 a protein.

In the instances where the protein is functionalized with at least one amino-oxy group, the polysaccharide, oligosaccharide, or carbohydrate contains at least one carbonyl group. The carbonyl groups may be a natural part of the polysaccharide structure, e.g., the reducing end of the polymer, or created, for
10 example, by oxidation. Reductive amination has been widely used to produce protein-polysaccharide conjugates. As a result, means to produce carbonyl-containing polysaccharides are well-known to those versed in the art.

Some polysaccharides contain a reducing sugar on their end, e.g., Hib PRP and Neisseria PsC. These contain aldehydes as hemiacetals and can be
15 reacted with amino-oxy reagents. Additional aldehydes may be created by specific degradation of the polysaccharide. General procedures are described in, for example, Lindberg et al. "Specific Degradation of Polysaccharides - Adv in Carbohydrate Chemistry and Biochemistry," Tipson et al., eds. Vol 31, pp. 185-240 (Academic Press, 1975). For example, when PRP is oxidized with sodium
20 periodate, the polysaccharide chain is cleaved so as to produce oligosaccharides with an aldehyde on each end.

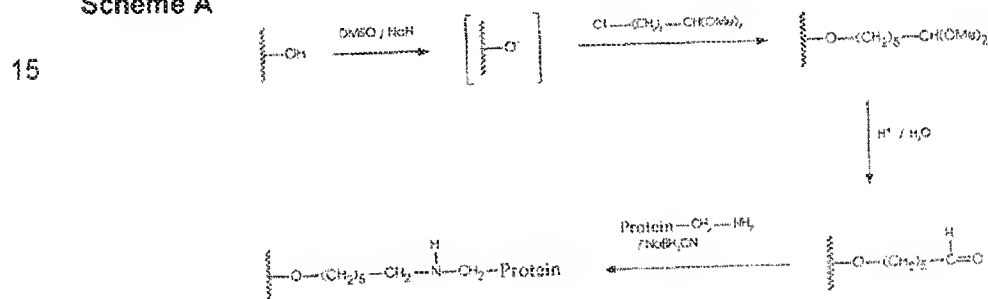
Many other methods for creating aldehydes are known to those versed in the art. For example Jennings et al., U.S. Patent No. 4,356,170 entitled "Immunogenic Polysaccharide-Protein Conjugates"; Tai et al., U.S. Patent No.
25 5,425,946 entitled "Vaccines against Group C Neisseria Meningitidis"; Porro, U.S. Patent No. 5,306,492 entitled "Oligosaccharide Conjugate Vaccines"; Yang et al., U.S. Patent No. 5,681,570 entitled "Immunogenic conjugate molecules";

Constantino et al., "Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C," *Vaccine* 10:691 (1992); Laferriere et al., "The synthesis of *Streptococcus pneumoniae* polysaccharide-tetanus toxoid conjugates and the effect of chain length on immunogenicity," *Vaccine*, 15:179 (1997).

In accordance with the present invention, it may be desirable to add aldehyde moieties to proteins and/or polysaccharides. Those of ordinary skill in the art will appreciate that there are many acceptable methods for doing so. Suitable non-limiting examples of methods to add aldehydes to proteins and polysaccharides include the following:

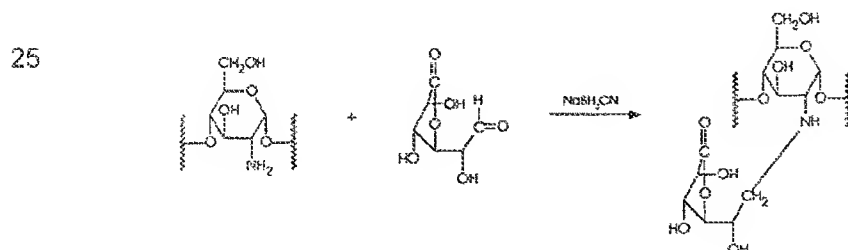
1. Hydroxyl groups are reacted with chlorohexanol dimethyl acetal in a base, and the masked aldehyde is subsequently revealed by mild acid hydrolysis. Dick et al., *Conjugate Vaccines* (Eds. Cruse, et al.), pp. 91-93 (1989).

Scheme A

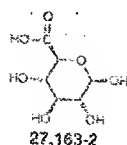
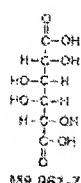


2. Glucouronic lactone and sodium cyanoborohydride are used to reductively aminate protein amines. Saponification is used to open the lactone. The sugar is then oxidized to an aldehydes using sodium periodate.

Scheme B



3. A carboxylated carbohydrate, for example, glucuronic acid, galactaric acid, glyceric acid, or tartaric acid is added to protein amines using a carbodiimide reagent. The glycosylated protein is then oxidized to create aldehyde moieties
- 5 using sodium periodate.

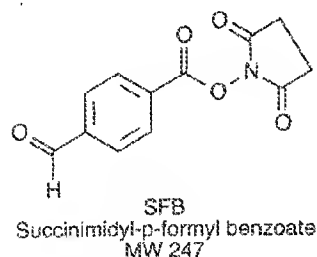


- 10 Galactaric acid Glucuronic acid

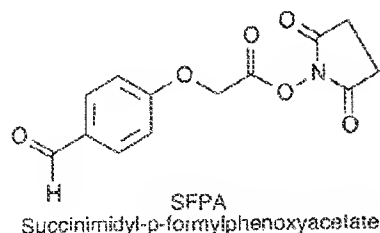
4. Aldehydes can also be created via enzymatic oxidation, using suitable oxidizing enzymes such as, for example, glucose oxidase, galactose oxidase, and neurominidase. For example, neurominidase may be used to remove terminal sialic acid, followed by galactose oxidase. (Hermanson, *Bioconjugation*
- 15 *Techniques*, p. 116-117).

5. Chemical addition of aldehydes to amines on proteins or polysaccharides can be effected using succinimidyl-p-formyl benzoate or succinimidyl-p-formylphenoxyacetate. These NHS esters of aldehydes react with amines and result in the addition of an aldehyde.

20



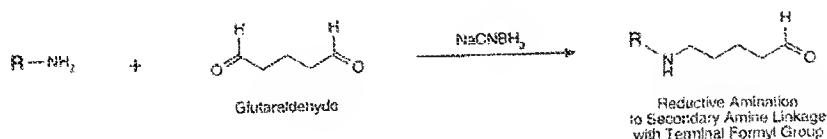
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6. Still another method uses the reaction of a bis-aldehyde (e.g., glutaraldehyde) with an amine. (Hermanson, *Bioconjugation Techniques*, p. 119-120).

Scheme C

5



7. Another suitable process is the addition of glyceraldehydes to protein
10 amines using reductive amination, followed by oxidation with sodium periodate to create aldehydes.

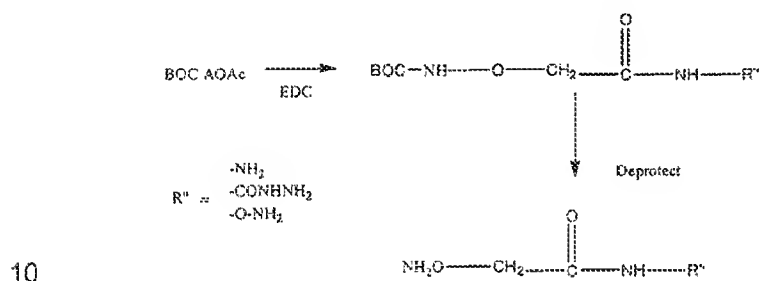
Optionally, if the conjugate contains residual free amino-oxy groups or aldehydes, and if it is desired to quench these groups, an additional step may be taken. One of the methods for quenching a conjugate having an aldehyde is by
15 reduction, e.g., using sodium borohydride. Alternatively, residual carbonyls may be quenched with a mono amino-oxy reagent, e.g., amino-oxy-acetate. Residual amino-oxy groups can be quenched with a monofunctional carbonyl, e.g., glyceraldehyde, acetone or succinic semialdehyde.

E. Amino-Oxy Reagents

20 The preparation of conjugate vaccines may be accomplished by the use of various amino-oxy reagents. A variety of useful homofunctional and heterofunctional amino-oxy reagents may be prepared by one skilled in the art, and may also be obtained from Solulink, Inc.TM, 9853 Pacific Heights Blvd., Suite H, San Diego, California 92121, and still others are described in the literature.
25 Many more can be conceived of and easily synthesized. Toyokuni et al., "Synthesis of a new heterofunctional linker, N-[4-(amino-oxy)butyl]maleimide for

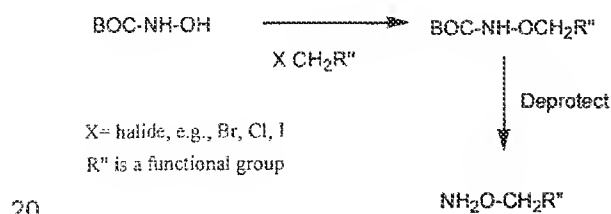
facile access to a thiol-reactive ^{18}F -labeling agent." *Bioconjugate Chem.* 14:1253 (2003).

Suitable non-limiting examples of reagents that may be used in accordance with the present invention include those prepared by Solulink™ (San Diego, California). For example, bis(amino-oxy)cystamine is a homofunctional amino-oxy-reagent that can be converted to a heterofunctional thiol-amino-oxy reagent. "Boc" is the art-recognized acronym for the t-butoxy carbonyl protecting group. Boc-amino-oxy acetate can be used to synthesize a number of suitable amino-oxy reagents according to, for example, the following scheme:

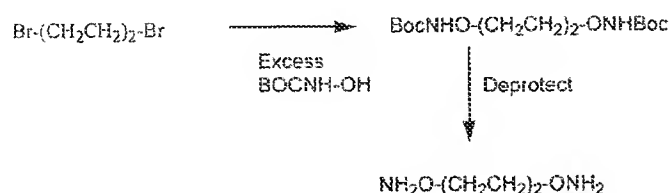


The ligands identified by R'' are suitable, non-limiting examples of nucleophilic ligands that may be used in accordance with the present invention.

The above reagents are based on 2-(Boc-amino-oxy) acetic acid, available from Bachem (Prod. No. A4605.005). Other useful starting reagents for making amino-oxy reagents include N-Boc-hydroxylamine and N-Fmoc-hydroxylamine. These reagents are available from Aldrich Chemical. N-Boc-Hydroxylamine can be used to prepare a useful amino-oxy reagent as follows:



Homofunctional amino-oxy reagents may be used in accordance with the present invention. Suitable homofunctional amino-oxy reagents that may be used include, for example, bis(amino-oxy)ethylene diamine, bis(amino-oxy) butane, and
 5 bis(amino-oxy)tetraethylene glycol, all of which are known and can be prepared by art-recognized methods. For example, bis(amino-oxy)butane may be prepared as follows:



10 Synthesis of various useful heterofunctional amino-oxy reagents have been described in the literature, for example Mikolajczyk et al., *Bioconjugate Chem.* 5:636 (1994) (a maleimide-amino-oxy reagent); Mikola & Hanninen *Bioconjugate Chem.* 3:182 (1992) (amino-oxy alkylamines); Webb & Kaneko *Bioconjugate Chem.* 1:96 (1990) (amino-oxy- dithionitropyridyl reagents). Jones et al. describe
 15 the synthesis of amino-oxy ethers from N-Boc hydroxylamine and alkyl iodides and bromides, which provide another route to useful amino-oxy reagents. Dixon & Weiss, *J. Org Chem.* 49:4487 (1984), describe bis-amino-oxy reagents that may be used in accordance with the present invention.

 Ketones may be added to amines using, for example, reagents like NHS
 20 levulate (from Solulink™). Carbohydrate groups on a protein, e.g., glycoproteins, can be oxidized to carbonyls with, for example, sodium periodate. In addition, reverse proteolysis may be used to add carbonyls or amino-oxy groups as described in Rose et al., "Preparation of well-defined protein conjugates using enzyme-assisted reverse proteolysis," *Bioconjugate Chem.* 2:154 (1991). N-